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Note

Sensitive monitoring of hexosamines in high-performance liquid chromatography by fluorimetric postcolumn labelling using the 2,4-pentanedione–formaldehyde system

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In a series of studies on carbohydrate analysis we have established conditions for automated analysis of hexosamines by high-performance liquid chromatography (HPLC) with postcolumn labelling by using 2-cyanoacetamide[1]. This monitoring system is convenient, because the derivatized products may be detected not only photometrically but also fluorimetrically, and also because it may be applicable to aldoses[2] and uronic acids[3]. However, its sensitivity (the lower limit of detection at signal-to-noise ratio 2 is 1.9 nmol for glucosamine and 3.4 nmol for galactosamine) is not sufficiently high for the small amounts of hexosamines present in biological and clinical samples to be analysed. Therefore, we have developed a more sensitive method by applying the Hantzsch reaction.

MATERIALS AND METHODS

Chemicals

A reagent-grade sample of 2,4-pentanedione was obtained from Kishida Kagaku (Osaka, Japan). A reagent-grade sample of formalin, also from Kishida Kagaku, was used as the source of formaldehyde. The authentic specimens of glucosamine hydrochloride was purchased from Wako (Osaka, Japan), and that of galactosamine hydrochloride was a gift from Dr. Hideo Kushida, Zeria Kogyo. The samples of shark cartilage chondroitin 6-sulphate and hog intestine heparin were obtained as sodium salts from Seikagaku Kogyo (Tokyo, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. Hen's egg albumin and bovine submaxillary mucin were from Sigma (St. Louis, MO, U.S.A.).

Acid hydrolysis of glycoconjugates

A sample of a glycoconjugate containing *ca.* 100 nmol of total hexosamine(s) was dissolved in 4.0 *M* hydrochloric acid (100 μl) in a glass ampoule, which was flushed with nitrogen for a few minutes, sealed and heated for 6 h at 100°C. The ampoule was cooled to room temperature and opened. A 1:1 (v/v) mixture (50 μl) of

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glycerol and ethanol was added to the hydrolysate, and the mixture was evaporated to dryness under reduced pressure by placing the tube in a desiccator containing sodium hydroxide pellets. The residue was dissolved in water (100 μl), and a 20-μl portion was injected into the HPLC column.

Instrumentation

A Hitachi 638 high-performance liquid chromatograph was used. A jacketed stainless-steel column (25 cm × 4 mm I.D.) was packed with Hitachi 2617 resin (spherical resin of polystyrene sulphonate type; average diameter 5.3 μm), as described in the previous paper[1], and eluted at 60°C with 0.040 *M* sodium tetraborate adjusted to pH 7.5 with concentrated hydrochloric acid, at a flow-rate of 0.30 ml/min. Samples were loaded onto the column via an injector carrying a 20-μl loop.

Procedure

To the eluate was added, via a Y-shaped PTFE connector, 6% 2,4-pentanedione in 0.10 *M* acetate buffer (pH 4.8) supplied from one pump-head of Atto 2396 twin-piston pumps at a flow-rate of 0.50 ml/min, and to the mixture was further added, in a similar manner, 9% formaldehyde in the same buffer pumped by the other head at the same flow-rate. The final mixture reacted in a PTFE coil (10 m × 0.5 mm I.D.) immersed in a glycerol bath thermostated at 95 ± 1°C. The reaction

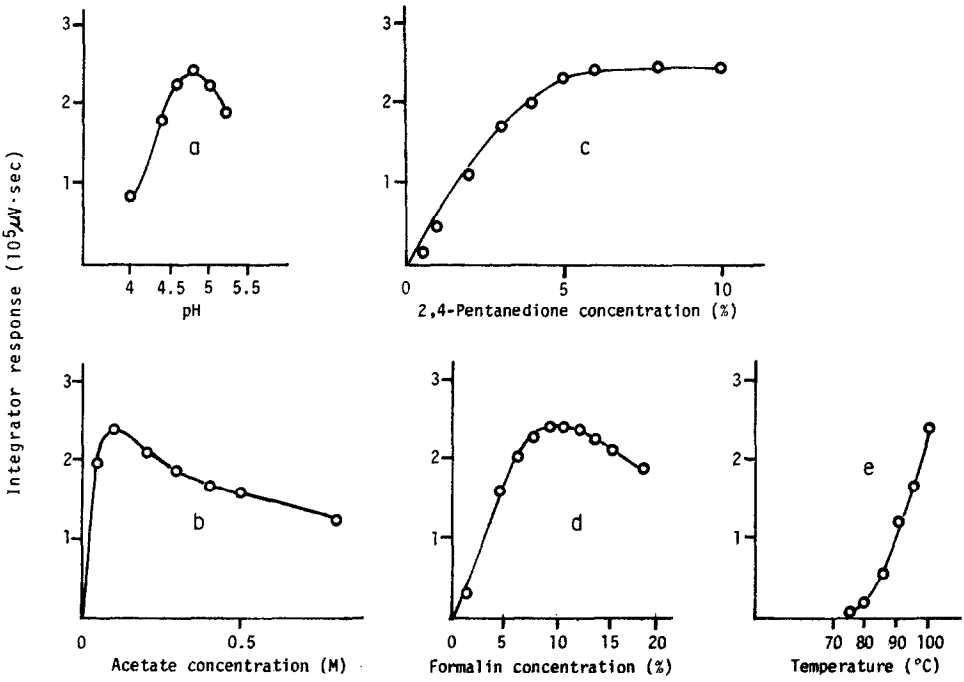

Fig. 1. Optimization of reaction condition for postcolumn labelling by the 2,4-pentanedione–formaldehyde system. Sample: glucosamine (2 nmol). (a) pH dependence of reagent buffer (acetate buffer) at the acetate ion concentration of 0.10 *M* (b) Effect of the concentration of the acetate ion of reagent buffer at pH 4.8. (c) Effect of the concentration of 2,4-pentanedione. (d) Effect of the concentration of formaldehyde. (e) Temperature dependence. For details of labelling procedure see text.

mixture was cooled to room temperature by passing it through a 1-m PTFE tube with the same bore size, and led into a Hitachi 650-10-LC fluoromonitor equipped with a 90-μl quartz flow-cell. Hexosamines were monitored at 417 (excitation) and 476 (emission) nm. In the optimization studies, samples were introduced directly into this derivatization system without passing through the column, and processed in the same manner.

RESULTS AND DISCUSSION

Reaction conditions were optimized in flow-analysis mode by treating glucosamine as a model with 2,4-pentanedione and formaldehyde, as described under *Procedure*. Glucosamine, as well as galactosamine, fluoresced with excitation and emission maxima at 417 and 476 nm, respectively. Fig. 1 summarizes the results.

Comparison of various kinds of buffer, including phosphate, borate, glycine and acetate, indicated that acetate buffer is the best suited for this reaction. The curves showing the effects of pH (a) and concentration (b) of acetate buffer gave the maximal intensities of fluorescence at pH 4.8 and 0.10 *M*, respectively. On the other hand, fluorescence intensity reached plateaux at 6% 2,4-pentanedione (c) and 9% formaldehyde (d). This reaction was very slow below 70°C, but speeded up at higher temperatures (e). However, temperatures lower than 100°C were advantageous for stabilizing the baseline. The most appropriate conditions were the use of 6% 2,4-pentanedione in 0.10 *M* acetate buffer (pH 4.8) and 9% formaldehyde in the same buffer at 95°C. Under these conditions 20–500 pmol of hexosamines could be determined with high reproducibility.

We applied these conditions to the postcolumn labelling of hexosamines separated on a Hitachi 2617 column with 0.040 *M* sodium tetraborate adjusted to pH

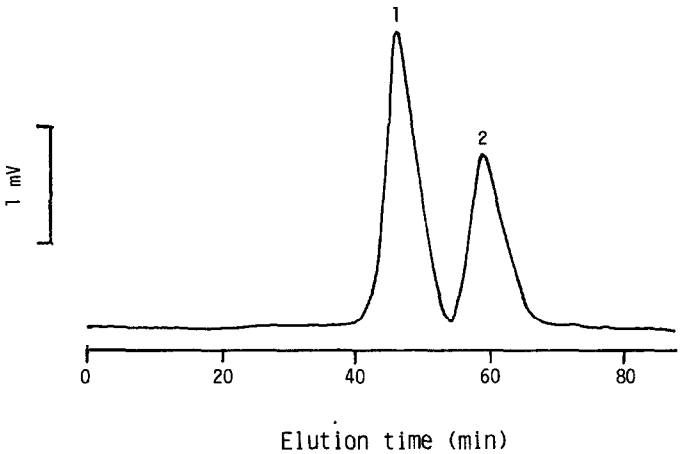

Fig. 2. Elution curve of an equimolar mixture of glucosamine and galactosamine, as monitored by using the 2,4-pentanedione–formaldehyde system. Peaks: 1 = glucosamine; 2 = galactosamine. Column, Hitachi 2617 (25 cm × 4 mm I.D.); column temperature, 60°C; eluent, 0.040 *M* sodium tetraborate adjusted to pH 7.5 with concentrated hydrochloric acid; flow-rate, 0.30 ml/min; sample scale, 10 nmol each; reagent solutions for postcolumn labelling, 6% 2,4-pentanedione in 0.10 *M* acetate buffer (pH 4.8, 0.50 ml/min) and 9% formaldehyde in 0.10 *M* acetate buffer (pH 4.8, 0.50 ml/min); reaction temperature, 95°C; wavelengths for detection: 417 (excitation) and 476 (emission) nm.

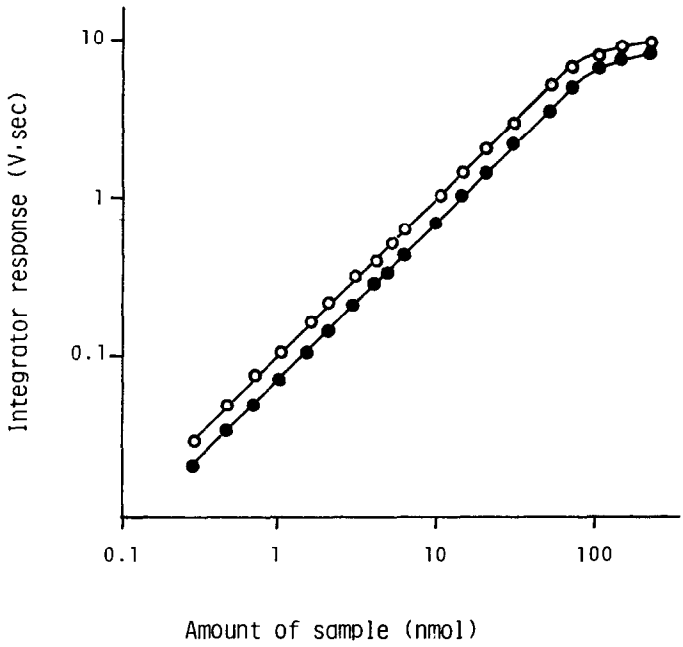

Fig. 3. Calibration curves of glucosamine (○—○) and galactosamine (●—●). The analytical conditions were the same as those described in Fig. 2.

7.5 with concentrated hydrochloric acid, the conditions used for the previous work[1]. The pH increase due to addition of this eluate was slight, the pH value of the reaction mixture being *ca.* 5.0. Therefore, the reduction of fluorescence intensity was negligible.

Fig. 2 shows the elution profile obtained for an equimolar mixture of glucosamine and galactosamine by the present method. The relative molar response of galactosamine to glucosamine was 0.71. Under these conditions the lower limits of detection for glucosamine and galactosamine were 140 and 230 pmol, respectively, at the signal to noise ratio 2. They both were lower than those in the 2-cyanoacetamide method by approximately one order of magnitude. Sharper separation will give higher sensitivity.

The calibration curves in Fig. 3 indicate good linearity for samples ranging from 0.3 to 70 nmol with both hexosamines. The coefficient of variation of the determination of glucosamine was 2.4, 1.5 and 1.7% at the 1, 10 and 70 nmol levels, respectively. The respective values for galactosamine were 2.4, 2.3 and 4.1%.

Although the present method is also positive for aliphatic amines other than hexosamines, most amines were eluted earlier than hexosamines. All amino acids were eluted in less than 15 min, thus did not interfere with the hexosamine analysis. Therefore, the present method is suitable for the analysis of component hexosamines in glycoconjugates. Fig. 4 shows some examples of such application. The samples of glycoconjugates were hydrolysed under the same conditions (6 h in 4 *M* hydrochloric acid at 100°C under a nitrogen atmosphere) and evaporated after addition of glycerol, as described in the previous paper[1], but the sample scale was reduced to *ca.* 10%.

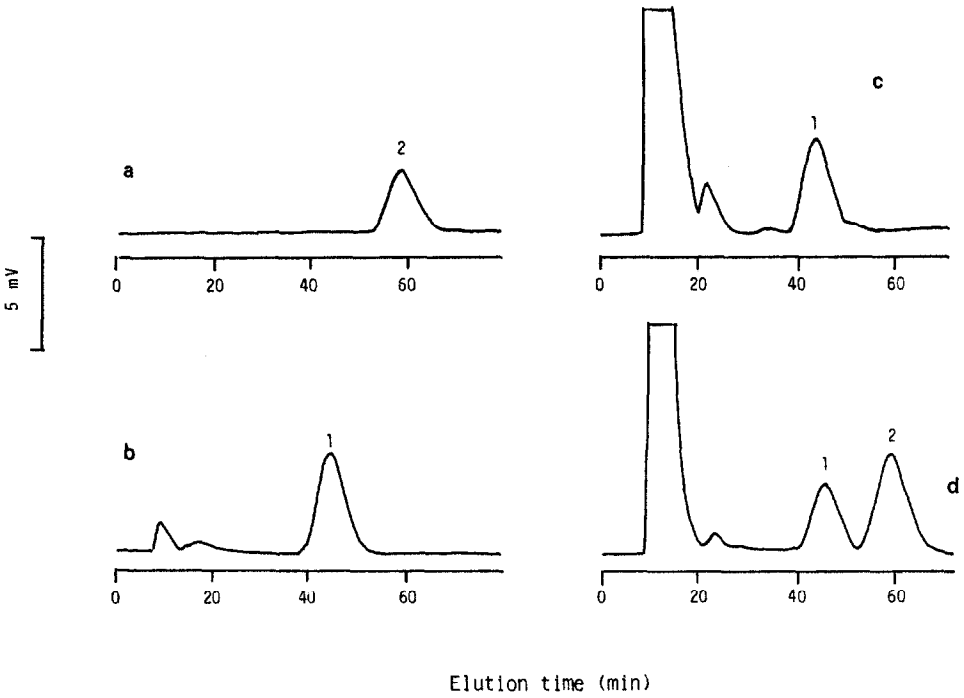

Fig. 4. Analysis of the acid hydrolysates of (a) shark cartilage chondroitin 6-sulphate, (b) hog intestine heparin, (c) hen's egg albumin and (d) bovine submaxillary mucin. For details of hydrolysis procedure see Experimental. The analytical conditions were the same as those described in Fig. 2.

All the hexosamine contents obtained were well consistent with the reported values[1].

Thus, the foregoing results demonstrate the usefulness of the present method involving the Hantzsch reaction, especially in hexosamine analysis of glycoconjugates. There are available a few other methods for fluorimetric postcolumn labelling of amines and/or amino acids, including the *o*-phthalaldehyde[4], fluorescamine[5] and pyridoxal–zinc[6] methods. Although none of them has been applied to hexosamine analysis, their sensitivities to hexosamines will be approximately of the same degree as the present method, if applied. However, it is an advantage of the present method that the reagents employed are much cheaper than those in other methods.

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